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STUDIES WITH ALKYLATING ESTERS—I
THE FATE OF ETHYLENE DIMETHANESULPHONATE

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Abstract—The fate of ^{14}C- and ^{35}S-ethylene dimethanesulphonate (EDS) has been studied in the mouse, rat, rabbit and monkey and compared with that of Myleran, a homologue of EDS.

The rat and mouse excreted mainly unchanged ^{35}S-EDS, whereas from the rabbit and monkey only a minor proportion of the unhydrolysed ester was recovered in the urine. Methane sulphonic acid was the only radioactive urinary metabolite, and in this respect no species variation was encountered. In the rat 1,2-^{14}C-EDS gave no evidence of simple hydrolysis since ethylene glycol was not found as a urinary metabolite. Expired radioactive carbon dioxide accounted for 5–8 per cent of the dose. Tissue distribution studies in the mouse show bone, blood and spleen have some selective uptake of EDS; the compound is only slowly cleared from these tissues.

The relevance of the findings are discussed and related to comparable studies with Myleran in relation to the biological activity of both compounds.

INTRODUCTION

OF THE homologous series of sulphonoxyalkane diesters (1) developed from the nitrogen mustards as anti-tumour agents,[1, 2] members $n = 4$ (Busulphan, "Myleran")[3] and $n = 9$ (Nonane)[4] have found clinical application in the treatment of certain leukaemias.

$$CH_3SO_2O\cdot(CH_2)n\cdot OSO_2CH_3 \qquad (1)$$

All members of the group possess activity against a variety of implanted tumours in animals with a maximum potency when $n = 4$ or $n = 5$. The effectiveness of these two members has been tentatively explained on a chemical basis.[5]

Ethylene dimethanesulphonate (EDS,1,$n = 2$), although a member of the series with low chemical reactivity, has moderate anti-tumour activity.[6] Unlike the other bifunctional sulphonoxyalkanes of this series,[7–9] it produces no naematological effects on animals even when near-lethal doses are administered. This segregates EDS from its homologues which possess the ability to cause characteristic depression of the granylocyte cell count in a variety of species,[7] following suppression of bone marrow activity.

The antifertility actions of these esters in male rats indicates that distinct effects are produced upon various cellular stages of spermatogenesis.[10] Ethylene dimethane-

sulphonate again differs from other sulphonic acid esters for its action is directed mainly at the intermediate range of spermatogenic cells, spermatids and spermatocytes.[11] This, together with its lack of effect on white blood cells, may imply a basic difference in mode of action. For this reason the present study was undertaken in the hope of finding biological determinants distinguishing EDS from other members of this series.

MATERIALS AND METHODS

Preparative methods

1,2-^{14}C-Ethylene dimethanesulphonate. Silver methanesulphonate (3·0 g) in acetonitrile (10 ml) was added to 1,2-^{14}C-ethylene dibromide (0·5 mc) in 1·4 g of inactive ethylene dibromide and the mixture heated in a sealed container at 120° for 16 hr. After cooling, the precipitated silver bromide was removed by filtration and washed with acetonitrile (20 ml). The combined filtrate and washings were concentrated *in vacuo* and the residual oil crystallised from chloroform and light petroleum ether (b.p. 40–60°) to give 1,2-^{14}C-ethylene dimethanesulphonate (1·2 g, 75 per cent) of specific activity 71μc/mM.

^{35}S-Ethylene dimethanesulphonate. This was prepared from ^{35}S-silver methanesulphonate (22 mc) using the method described above with a specific activity of 46 mc/mM. Both products (m.p. 45°) were homogenous by combined paper chromatography and autoradiography.

Chromatography

The ascending technique was used on Whatman No. 1 paper. Solvent systems were *n*-butanol:dioxan:2N ammonia (4:1:5) and *n*-butanol-2N acetic acid (1:1). Analysis of the chromatograms was carried out on a BTL paper strip scanner and also by contact autoradiography on Ilford X-ray film. Urine was chromatographed directly but chromatography of whole blood was unsatisfactory.

Radioactive assay

Samples were counted in an IDL Tritomat Liquid scintillation counter as well as by comparison with ^{35}S and ^{14}C standards on a BTL strip scanner. Tissues were prepared for scintillation counting by digestion and oxidation with perchloric acid and hydrogen peroxide. Tissues, or tissue aliquots up to 1 g, were taken from three mice pooled, weighed and transferred to a 20 ml McCartney bottle. Perchloric acid 70% (3 ml) and hydrogen peroxide 30% (2 ml) were added to each sample and the sealed bottles heated at 60–80° for 12 hr. Aliquots (usually 0·25 ml) were taken for counting in a phosphor solution of toluene and 2-ethoxyethanol (2:1) containing PPO (0·6%). Blood, plasma, urine and faeces samples were prepared for scintillation counting by the method of Mahin and Lofberg[12] using the same scintillant. Other materials were prepared in a toluene and Triton X-100 (2:1) scintillant containing PPO (0·4%) and POPOP (0·01%).

Collection of faeces, urine and expired gases

Animals were housed in metabolic cages with access to pellet food and water *ad libitum*. Urine was collected in a receiver cooled with carbon dioxide; faeces, collected separately, were air-dried. Where measurement of radioactive carbon dioxide in

expired gases was required, animals were housed in an all-glass metabolic container through which carbon dioxide-free air was drawn at a rate of 300 cm^3/min.

The respired gases were passed through a solution of sodium hydroxide (20%, 200 ml). Treatment of an aliquot (20 ml) of this solution, to which sodium carbonate (100 mg) was added, with hydrochloric acid allowed the liberated carbon dioxide to be trapped in "Hyamine hydroxide" (Nuclear-Chicago). An aliquot of the "Hyamine" solution was taken for radioassay.

Examination of urine for EDS and ethylene glycol

EDS was confirmed as the major radioactive component in animal experiments by recrystallisation of 1,2-^{14}C-EDS or ^{35}C-EDS from urines to constant specific activity: to urine (20 ml) was added EDS (400 mg) and the mixture continuously extracted with ether for 16 hr. The ethereal extract was evaporated to low volume, methanol added and the mixture filtered. Addition of petrol ether (40°–60°) gave needles of EDS which were filtered off and recrystallised to constant specific activity alternately from methanol, acetone and chloroform. The product had a m.p. of 45° undepressed by admixture with an authentic sample of EDS.

Labelled ethylene glycol was not detected as a potential urinary metabolite after administration of ^{14}C-EDS on recrystallisation of the bisphenyl urethane derivative from urine samples by the method of Gessner *et al.*[13]

Animals and administration of compounds

Rats were male Wistars (about 250 g) and mice (about 25 g) were males of an RF strain. Male Dutch rabbits (2–3 kg) and one female Rhesus monkey (2·5 kg) were used.

Compounds were administered either as a suspension in arachis oil or in 40% dimethyl sulphoxide in water. Drug pellets were prepared by mechanical compression of radioactive EDS with an equal weight of cholesterol and bilateral implants made subcutaneously in the interscapular region.

RESULTS

In all species examined the majority of the urinary radioactivity is excreted in the first 24 hr after treatment. Chromatographic examination of urine from animals receiving ^{35}S-EDS indicates the presence of two radioactive areas corresponding to EDS and methanesulphonic acid (MSA) with no indication of degradation to sulphate (Table 1). Rat and mouse urine collected in the first 24 hr after administration of ^{35}S-EDS contains a considerable amount of unchanged compound (Fig. 1) and even after 4 days unchanged EDS can still be detected in the urine. Over a wide range of doses and by different routes about the same percentage of EDS is converted to MSA in the mouse and rat (Fig. 1). This represents an appreciable amount of reaction as the half hydrolysis time for EDS in either plasma or physiological saline is 12 days at 37°. In contrast to other studies in the rat and mouse, rats implanted at the higher dose level (equiv. to 85 mg/kg) excreted 32 per cent of the implanted EDS as MSA. Like Myleran, the species toxicity increases in the order mouse, rat and rabbit. Single experiments in rabbit and monkey show a much higher percentage excreted than at comparable dose levels in the rat and there is also a much greater conversion to MSA (Fig. 1). This species difference is well illustrated by comparing an oral dose

TABLE 1. CHROMATOGRAPHIC DATA FROM URINE OF ANIMALS TREATED WITH ^{35}S-EDS

Compound	RF in solvent system			
	BA		BDN	
EDS	0·82	(0·84)	0·84	(0·85)
MSA	0·14	(0·16)	0·12	(0·12)
Sulphate	—	(0·00)	—	(0·00)

BA: *n*-Butanol, acetic acid.
BDN: *n*-Butanol, dioxan, ammonia.
Figures in parentheses refer to authentic samples.

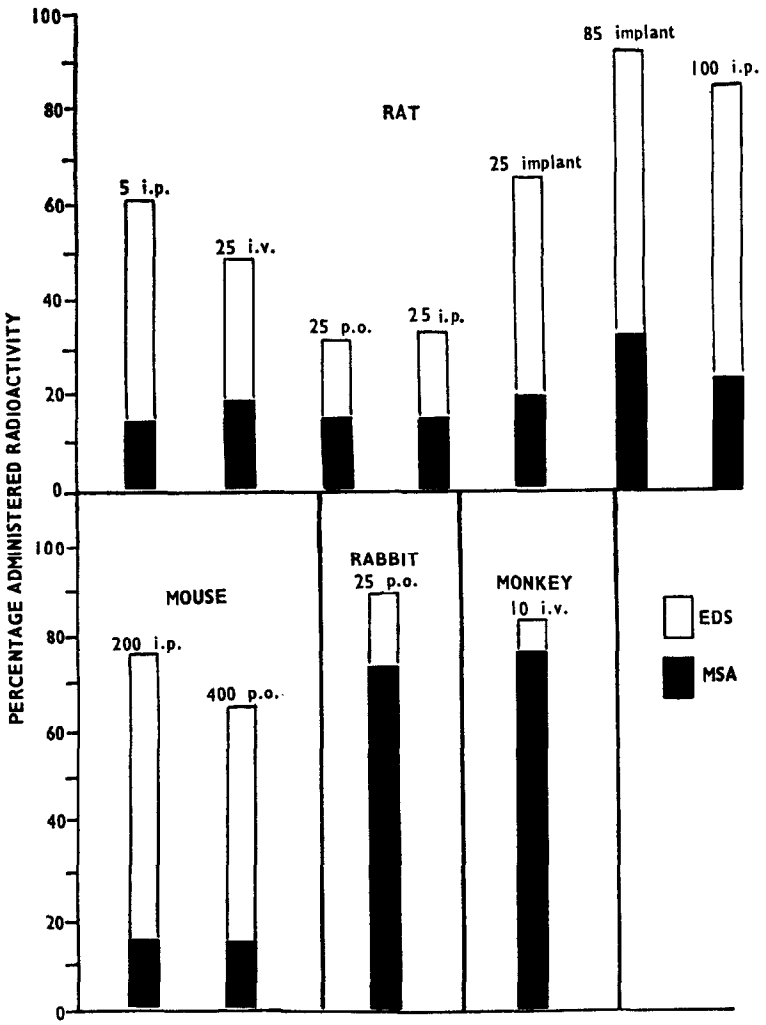

FIG. 1. Dose (mg/kg) and route of administration are given at the head of each column. The column height indicates the percentage of administered radioactivity excreted in 0–24 hr urine and the proportions of EDS and MSA are shown.

of 25 mg/kg in the rat and rabbit. Elimination through faeces is probably insignificant as most of the radioactivity (about 2 per cent of the dose) is removed by washing.

Preliminary studies in the rat and mouse with 1,2-^{14}C-EDS confirmed the high proportion of intact drug in the urine 24 hr after administration. Recovery of label in urines was up to 70 per cent of the dose and as over 60 per cent of the administered drug is excreted unchanged, this represents urinary metabolites corresponding to some 10 per cent of the dose. A small amount (5–8 per cent) of the dose could be accounted for as expired carbon dioxide in 24 hr. Ethylene glycol was not detected as a urinary metabolite of 1,2-^{14}C-EDS.

A comparison of levels of activity in mouse tissues after intraperitoneal administration of EDS labelled with ^{14}C and ^{35}S showed at 1 hr selective uptake of the drug by blood, spleen, stomach and bone, to a lesser extent by the testis and cauda epididymis (Table 2). By 3 hr distribution had become more uniform but at 24 hr levels of activity in blood was still appreciable. Plasma contained unchanged EDS up to 24 hr from a single dose but only a small amount of MSA is evident due to its rapid and almost quantitative elimination; over 90 per cent of a dose of ^{35}S-MSA being excreted unchanged by the rat in 8 hr.

Plasma radioactivity from rats receiving ^{35}S-EDS orally, parentally or by subcutaneous implant indicates slow removal of the drug from the blood but, surprisingly,

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN MOUSE TISSUES OF ^{14}C-AND ^{35}S-EDS

	Hours after administration of ^{35}S-EDS			Hours of administration of ^{14}C-EDS		
Tissue	1	3	24	1	3	24
Bone*	4·6	4·7	0·27	3·0	3·1	0·31
Brain	2·5	2·3	0·13	1·7	1·5	0·12
Cauda epididymis	4·2	1·4	0·32	3 4	5·3	0·71
Residue of animal	2·3	1·5	0·21	1·6	1·3	0·15
Fat	0·93	1·4	0·12	0·77	0·55	0·14
Heart	3·3	2·0	0·19	2·2	1·9	0·31
Large intestine†	2·1	1·5	0·11	1·5	1·3	0·01
Small intestine†	1·8	1·7	0·11	1·5	1·7	0·10
Kidney	1·3	1·2	0·08	2·3	2·8	0·17
Liver	2·5	0·26	0·17	1·0	0·54	0·17
Lung	2·6	3·2	0·17	2·6	2·3	0·77
Muscle‡	1·4	1·5	0·12	1·7	1·4	0·18
Skin	1·6	0·99	0·13	1·2	1·9	0·27
Spleen	6·2	6·1	0·15	4·2	3·0	0·53
Stomach	4·8	6·8	0·35	3·2	2·6	0·43
Testis	3·1	2·8	0·30	2·9	1·7	0·25
Whole blood	6·4	4·7	0·12	13·0	8·3	1·4
Plasma	3·5	1·2	0·08	5·3	8·2	0·35

Results are expressed as per cent administered dose per g wet tissue after i.p. dose 100 mg/kg in 40% aqueous dimethyl sulphoxide.

* Whole femur was used.

† Large and small intestines and stomach included their contents.

‡ Gatrocnemius muscle was used.

formulations in oil or in pellets have little effect in delaying its absorption from the site of administration.

DISCUSSION

In all four species examined in the present study, a proportion of ^{35}-S-EDS is excreted unchanged in the urine, the remaining radioactive material present being MSA.

Williams *et al.* report[13] that varying amounts of administered ethylene glycol are excreted unchanged by the rat and the rabbit and we have confirmed this with 1,2-^{14}C-ethylene glycol, of comparable specific activity to 1,2-^{14}C-EDS. However, dilution of 1,2-^{14}C-EDS urine with inactive ethylene glycol followed by extraction and formation of the bisphenyl uramide derivatives showed that ethylene glycol was not a urinary metabolite. It appears therefore, that little, if any, simple hydrolysis of EDS occurs *in vivo*. Thus the proportion of MSA excreted is probably a measure of the extent of detoxification and direct chemical reaction of the compound. Over a wide dose range (5–100 mg/kg), the pattern of ^{35}S-EDS metabolism in the rat is similar (Fig. 1) indicating that conversion to MSA is not dose dependent. The route of administration makes little difference to the proportionate recovery of radioactivity in the urine nor to the relative amounts of MSA and unchanged EDS.

In the rabbit and monkey the trend is to higher recovery of radioactive material with a much greater breakdown of the ester. It is interesting that the rabbit is the most susceptible to a lethal action from EDS, which may indicate a greater proportion of direct tissue reaction. The mouse tolerates much higher doses than the other species, in general agreement with the comparative toxicity of other sulphonoxyalkane diesters in experimental animals. Larger doses of EDS are required to produce pharmacological effects in the mouse and this is clearly not a reflection of more rapid metabolism (Fig. 1) but due to some innate resistance of the tissues susceptible to damage by this compound.

The contrast between the metabolic behaviour of Myleran and EDS is surprising. The former is largely degraded by the rat, up to 80 per cent of the ^{35}S-label appearing in the urine within 32 hr.[14] The main metabolite identified is MSA with a small proportion (up to 6 per cent) of unchanged drug; two other unidentified metabolites and a trace of sulphate are also present.[14, 15] By contrast, the administration of ^{35}S-EDS at a similar dose level (5 mg/kg) gives a high proportion of unchanged EDS excreted (Fig. 1). Studies of the distribution of the radioactive label following adminstration of ^{35}S-Myleran to rodents shows rapid disappearance from the circulation[16] and a comparable pattern has been demonstrated in man.[17] However, radioactivity from ^{35}S-EDS persists in the blood, mainly as unchanged drug. Attempts to reduce the absorption of the compound by using compressed implants prepared with equal amounts of EDS and cholesterol were not successful, the drug being rapidly removed from the depot. Other formulations are being examined so as to obtain a slow but sustained release of EDS *in vivo*. There is clearly no simple correlation between the prolonged availability of a diester of this series and its biological potency; for example Myleran, although metabolised rapidly, has a far greater cumulative potential than EDS and a much lower biologically effective dose level.[18]

Using ^{14}C-EDS the fate of the alkane segment of the diester *in vivo* can be determined. This alkylating moiety is almost certainly responsible for the biological

action of the compound. Metabolic studies confirm the relative excretion of unchanged EDS in the three species in agreement with the experiments using ^{35}S-EDS. Chromatographic separation of labelled compounds excreted in the urine of rats treated with ^{14}C-EDS indicates the presence of two metabolites besides unchanged EDS.[19] Neither labelled ethylene glycol nor β-hydroxyethyl-methanesulphonate are present suggesting that simple hydrolysis of the ester is not a major metabolic route. Between 5–8 per cent of the injected ^{14}C-EDS is expired as labelled carbon dioxide which compares with 12 per cent conversion of 1,4-^{14}C-Myleran to carbon dioxide,[20] so that complete oxidation of the carbon chain occurs only to a limited extent with either compound.

Tissue distribution patterns of radioactive material after administration of EDS indicate that the lack of observed functional impairment on any blood component is not a reflection of difference in radioactivity distribution (Table 2). ^{14}C and ^{35}S levels in the small intestine at 1 hr and 3 hr are similar, perhaps indicative of little biliary excretion of any metabolite produced in the liver. By contrast methyl methanesulphonate is largely metabolised in the liver,[21] S-methyl cysteine being excreted in the bile. Levels of activity at 24 hr indicate most of the activity associated with the intact drug with very little residual alkylation, and EDS of a much higher specific activity would be required to investigate the sites of cellular alkylation in detail .

Differences in the chemical reactivity of members of this sulphonoxyalkane series may possibly correlate with the type of cell affected. Both Myleran and methylene dimethanesulphonate (MDS, 1, $n = 1$)[10] act on early cell stages (e.g. spermatogonia). EDS, which is only slowly hydrolysed and is not so chemically reactive as Myleran or MDS, exerts its main pharmacological action on later cell stages in spermatogenesis (spermatocytes and spermatids). As assessment of the anti-tumour action of these diesters is difficult to analyse, but reasoning that these related esters attack different stages of cell proliferation and differentiation in tissue systems (as exemplified by the testis) combined treatment even with closely related compounds might provide more effective control of growth in certain tumours.

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